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*AN EARLY EFFECT OF ESTROGEN ON PROTEIN SYNTHESIS**

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In studies of the mechanism of action of estrogens, Mueller, Gorski, and Aizawa demonstrated that, when protein synthesis was blocked by puromycin, the increases induced by estrogen in phospholipid and ribonucleic acid synthesis were also blocked.[1] They concluded that uterine responses to estrogen are dependent on protein synthesis and pointed out that the elucidation of the nature of this protein synthesis is critical to understanding the mechanism of action of estrogen. Estrogen may stimulate protein synthesis in general by influencing, directly or indirectly, some component of the protein-synthesizing systems of the cell. This would result in the nonselective increase in synthesis of various types of proteins, including those necessary for further expression of the estrogen response. Another possibility is that estrogen brings about the *de novo* synthesis of a limited number of proteins necessary for the uterine response to estrogen. These proteins might in turn influence various metabolic pathways, including the protein-synthesizing systems themselves. It was impossible to distinguish between these two possibilities previously, because studies of the estrogen effect on protein synthesis were carried out at time periods of 4 hr or greater after estrogen administration when the rate of over-all protein synthesis is markedly stimulated.[1, 2]

In order to ascertain the nature of the protein synthesis stimulated by estrogen, we investigated the effect of estradiol-17β on *in vivo* protein synthesis in various cell fractions at 2 and 4 hr after hormone treatment. The relationship between RNA and protein synthesis in rats treated 2 hr with estrogen was studied, utilizing the puromycin inhibition of protein synthesis.

Experimental Procedure.—Immature female rats weighing approximately 50 gm were injected intraperitoneally with 5 μg estradiol-17β in 0.5 cc of 0.154 *M* NaCl (saline) at 2 and 4 hr prior to sacrifice. Control animals received 0.5 cc saline intraperitoneally 1 hr prior to sacrifice. Protein synthesis was determined by measuring the incorporation of H^3-leucine (2 μc/rat) or glycine-2-C^{14} (1 μc or 2 μc/rat) into protein, and RNA synthesis was determined by measuring the incorporation of H^3-cytidine (2 μc/rat) into RNA. All animals were injected with isotope in 0.5 cc saline 1 hr prior to sacrifice. After sacrifice the uteri were excised and homogenized in 5 ml cold homogenization medium.[3] The homogenates were centrifuged at 800 $\times$ *g* for 10 min. The supernate was decanted from each sample, and the residue resuspended in 2 ml homogenization medium and centrifuged at 800 $\times$ *g* for 10 min. The resulting residue is termed the nuclear fraction. The two supernates for each sample were combined and centrifuged at 15,000 $\times$ *g* for 15 min to obtain the mitochondrial fraction. The supernate was then centrifuged at 105,000 $\times$ *g* for 1 hr to obtain the ribosomal fraction. In certain cases, the supernatant fraction was further centrifuged for 3 hr at 105,000 $\times$ *g* to obtain a postribosomal pellet. The final supernate in all cases is termed the soluble fraction. Five ml cold 5% perchloric acid (PCA) was added to each residue, and 2 ml cold 25% PCA was added to each supernate. After centrifugation, the supernate was discarded, and the protein residue was washed 3 more times with 5 ml of 5% PCA. This was followed by successive washes of 95% ethanol, chloroform and ethanol (2:1), and 2 ether washes to remove lipid. After the last ether wash, the residue was suspended in 1 ml 0.05 *M* Tris buffer (pH 7.4) and 25 μg ribonuclease was added to liberate the RNA. The suspension was incubated for 2 hr at 37.5°C with occasional stirring. The reaction was then stopped with 1 ml 1.0 *N* HCl, and the samples were centrifuged. The supernate containing the mixture of ribonucleotides and oligoribonucleotides was evaporated to dryness under heat to hydrolyze the oligonucleotides. The residue was chromatographed on paper in an isobutyric acid:0.4 *N* NH_4OH (75:25) solvent system. The cytidine monophosphate (CMP) spots were eluted in 2 ml distilled water and quantitated by their ultraviolet absorption. Aliquots of the samples were counted in a Packard Tri-Carb scintillation counter.

The protein residues were rewashed once with PCA followed by the organic washes described above. The dried residues were then weighed and counted using the scintillation technique.

RNA polymerase in the nuclear fraction was assayed in a system similar to that described by Weiss.[4] Uterine nuclei were incubated 10 min at 37°C with 1 μc H^3-cytidine triphosphate (Schwarz BioResearch) in 1.0 ml of medium at pH 8.0 containing 100 μm Tris-Cl, 10 μm cysteine, 5 μm $MgCl_2$, 75 μm KCl, 30 μm NaFl, 1 μm ATP, 0.4 μm GTP, 0.4 μm UTP. At the end of the incubation, cold 5% PCA was added, and the nucleic acid-protein residue was prepared by the washing procedure described above (up to ribonuclease treatment). The dry residue was weighed, dissolved in hydroxide of hyamine, and counted in a scintillation counter. Data were calculated as counts per minute per mg of dry residue.

Animals receiving puromycin injections were injected with 5 mg puromycin dihydrochloride in 0.5 cc saline 15 min before estrogen administration. A second injection of the same amount of puromycin was made 1 hr after the first puromycin injection. Animals not receiving puromycin were injected with a control saline solution.

Results.—Figure 1 summarizes data from 3 experiments in which the incorporation of glycine-2-C^{14} into protein of various cell fractions was determined. No significant effect of estrogen on protein synthesis was detectable in nuclear, mitochondrial, ribosomal, or soluble fractions of the rat uterus at 2 hr after estrogen administration. However, 4 hr after estrogen, a marked increase in protein synthesis could be observed in all particulate fractions. The specific activity of the soluble fraction increases only slightly at 4 hr and was no greater than at 2 hr. A comparison was then made between H^3-leucine and glycine-2-C^{14} incorporation by injecting the isotopes in combination and assaying the protein at 2 and 4 hr. The data compiled in Table 1 show that both amino acids exhibit the same pattern of incorporation into the cell fractions. Again, the effect of estrogen is virtually lacking at 2 hr, while at 4 hr the specific activities of the proteins of the various

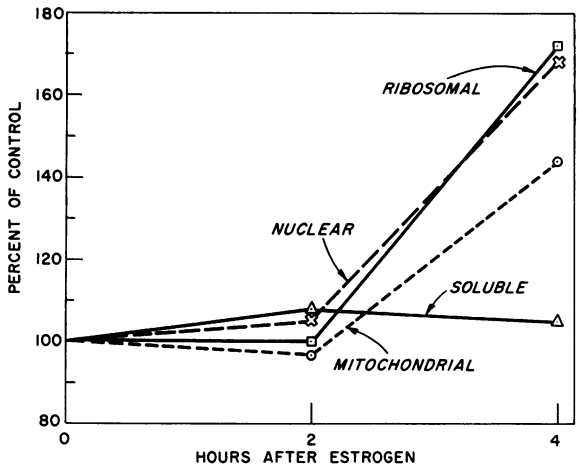

FIG. 1.—Effect of estradiol on protein synthesis in subcellular fractions. 5 μg estradiol injected intraperitoneally at times indicated. 1 μc of glycine-2-C^{14} injected 1 hr prior to killing. Each point represents average of 6 groups from 3 separate experiments. Each group used pooled uteri from 6 rats, making a total of 36 rats represented for each time point. Data are expressed as percentage of control specific activities, and methods are as described in text.

cell fractions were increased by estrogen 47 to 239 per cent above control values. In contrast to the previous experiments, the soluble fraction appears to be affected by estrogen at 4 hr. The degree of this response, however, was much smaller than in the particulate fractions.

Since an increase in protein synthesis in response to estrogen could not be detected at 2 hr, it was not considered necessary to separate each particulate fraction in determining the effects of puromycin on protein and RNA synthesis at 2 hr in the experiment shown in Figure 2. When H^3-cytidine and glycine-2-C^{14} were injected in combination, no estrogenic stimulation of protein synthesis was observed at 2 hr.

TABLE 1

EFFECT OF ESTRADIOL ON PROTEIN SYNTHESIS IN SUBCELLULAR FRACTIONS OF THE RAT UTERUS

Cell fraction	Incorporation of Glycine-2-C^{14} into Protein — Hr after estrogen			Incorporation of H^3-Leucine into Protein — Hr after estrogen		
	0	2	4	0	2	4
	Specific activity (DPM/mg)			Specific activity (DPM/mg)		
Nuclear	177	171	356	283	223	452
	174	176	415	185	244	576
Mitochondrial	349	346	1,028	423	573	861
	390	334	859	479	443	957
Ribosomal	422	616	1,359	747	952	1,773
	542	691	1,913	802	976	2,031
Postribosomal	939	739	1,220	1,524	1,044	1,668
	782	749	1,527	1,020	1,108	2,053
Soluble	705	785	1,114	819	1,074	1,337
	708	695	1,239	886	903	1,594

A mixture of 2 μc glycine-2-C^{14} and 2 μc H^3-leucine was injected intraperitoneally into each rat. Two groups consisting of six animals were used for each time period. The first value listed for each fraction was determined from the same group of animals. Techniques used for fractionation of the cells and the determination of specific activity are described in the text.

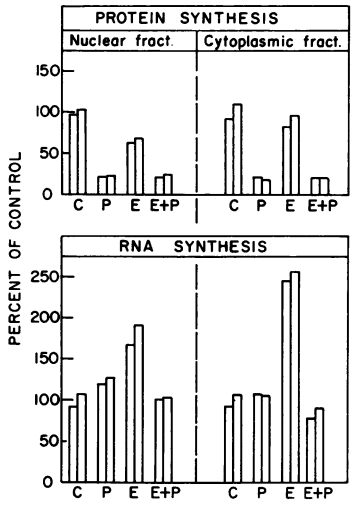

FIG. 2.—Effect of puromycin on synthesis of protein and RNA in uteri from control and 2 hr estrogen-treated rats. Rats received 1 μc glycine-2-C^{14} and 2 μc H^3-cytidine 1 hr prior to killing. 5 mg puromycin or saline (in controls) was injected hourly, starting 15 min prior to estrogen administration. Nuclear fraction separated as described in text, and supernate designated as cytoplasmic fraction. Each bar represents uteri pooled from 3 rats. Procedures for protein and RNA assay described in text. Data expressed as percentage of control specific activity. C = control; P = control plus puromycin; E = estrogen; E + P = estrogen plus puromycin.

In contrast to the lack of stimulation of protein synthesis at 2 hr, estrogen did increase the synthesis of RNA in both nuclear and cytoplasmic fractions (Fig. 2). Specific activities were considerably higher in nuclear fractions as compared to cytoplasmic fractions, and support previous observations on orthophosphate-P^{32} incorporation into RNA.[1, 5]

Administration of puromycin reduced protein synthesis in both estrogen-treated and nontreated groups to 20 per cent of the control values. At the same time, puromycin blocked the estrogen effect on RNA synthesis at 2 hr in both nuclear and cytoplasmic fractions, while it had no significant effect on RNA synthesis in controls. The results reported here, as well as those from a previous study,[5] indicated that estrogen causes a rapid increase in RNA synthesis. We directed our attention therefore to the nuclear enzyme, RNA polymerase, as a possible rate-limiting step controlled by estrogen.

Figure 3 shows that RNA polymerase had approximately 100 per cent greater activity in the 2 hr estrogen-treated rats as compared to controls. Puromycin blocks the increased activity due to estrogen, but does not have an effect on enzyme activity in the controls. Details of the characterization of uterine RNA polymerase and its reponse to estrogen administration will be reported elsewhere.

Discussion.—The results show that at 4 hr the increase in protein synthesis due to estrogen is a generalized response occurring in all cell fractions except possibly the soluble fraction. At this time, apparently the activity of the protein-synthesizing

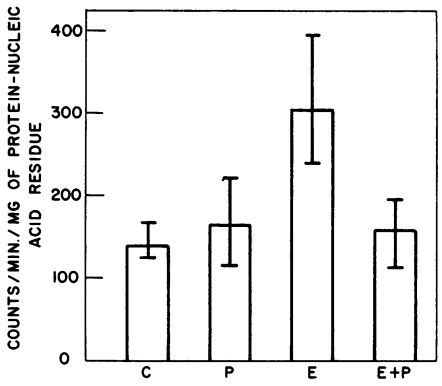

FIG 3.—Effect of estradiol and puromycin on activity of uterine RNA polymerase. Rats injected with 5 μg estradiol-17β 2 hr prior to killing. 5 μg puromycin administered 15 min prior to estradiol and again 1 hr later. RNA polymerase assay as described under *Experimental Procedure*. Each bar represents average of 4 determinations from 2 nuclear preparations, each representing 3 pooled uteri. Brackets indicate range of 4 values. C = control; P = control plus puromycin; E = estrogen; E + P = estrogen plus puromycin.

systems of all the subcellular organelles has increased due to estrogen, or else the newly synthesized proteins from one type of organelle have migrated throughout the cell, presenting the appearance that all fractions have increased synthetic activity. Recently, similar increases in the accumulation of newly synthesized RNA and phospholipid in all cell fractions within 2 hr after estrogen treatment have been observed in this laboratory.[5] However, as can be seen from the data presented here, there is no detectable estrogen response in protein synthesis at this earlier time. The late appearance of the estrogen response in protein synthesis indicates that the protein synthesis observed at 4 hr could well be dependent on the prior estrogen-stimulated increase in RNA synthesis.

A most important aspect of the results is the lack of a detectable estrogen effect on protein synthesis in general in any fraction at 2 hr, while at the same time, the RNA synthesis stimulated by estrogen is puromycin-sensitive. If we assume that puromycin is only inhibiting protein synthesis, then estrogen must bring about the synthesis of protein necessary for the increase in RNA synthesis.

Demonstration that estrogen causes an increase in RNA polymerase activity which is also blocked by puromycin suggests that this enzyme activity may be responsible for the effects we have observed with respect to RNA synthesis. It cannot be concluded at this time, however, whether estrogen is influencing this activity by directly increasing the rate of synthesis of polymerase or synthesis of some other protein which influences polymerase activity.

Other work in this laboratory[6] has shown that when uniformly labeled C^{14}-glucose was incubated with uteri *in vitro*, estrogen significantly stimulated the incorporation of the glucose into protein, RNA, and lipid by 1 hr. Again, this effect was blocked by puromycin.

We are led therefore to the conclusion that one of the earliest estrogen effects is the induction of synthesis of a small group of proteins which are essential for the

elaboration or amplification of initial estrogen action, and that the increased metabolic activity of the uterus due to estrogen action can be attributed to its influence on the production of these proteins. The total number of these proteins must represent such a small percentage of the total protein as not to be detectable by measuring incorporation of labeled amino acids into protein.

It now becomes necessary, in studying the mechanism of action of estrogen, to explain how the interaction of estrogen with a site in the cell results in the selective synthesis of these proteins. Postulations that estrogens function by directly activating the protein-synthesizing systems fail to explain the selective synthesis of proteins and the lag in estrogen effects on protein synthesis that occurs during the first 4 hr. A more likely possibility is that estrogen interacts with a component of the cell which has the capability of controlling the type of protein synthesized. Such a mechanism may be similar to that outlined by Jacob and Monod[7] which supposedly regulates enzyme induction in bacterial systems. However, the role of estrogen in such a system cannot be explicitly defined. While estrogens might act directly as regulators of genetic expression, any hypothesis for estrogen action must take into consideration the possibility that estrogens may function in more remote steps which in turn result in the production of the actual regulators. The difficulty in attacking the estrogen problem has been that the diversity of secondary effects has obscured the initial effects which occur within an extremely short period of time after hormone administration. In view of the apparent estrogen action on specific protein synthesis, further investigations in this area will be necessary to explain the mechanism of estrogen action.

Summary.—A marked increase in incorporation of labeled amino acids into proteins of all particulate fractions of the rat uterus occurs 2–4 hr after estrogen administration. No effect on incorporation into portein can be detected at 2 hr. At 2 hr, RNA synthesis and the activity of uterine RNA polymerase are increased, and these increases are blocked when protein synthesis is inhibited by puromycin. It is concluded that the increased synthesis of specific proteins plays an important role in the early action of estrogen and is followed by an increase in the over-all synthesis of protein.

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